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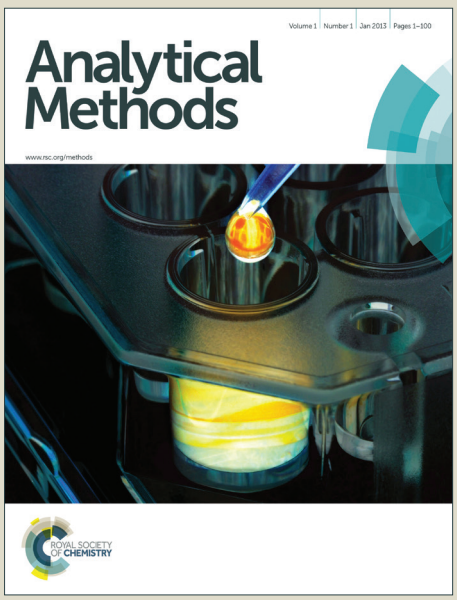

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L-lysine derived organogelator-based stationary phase for mixed-mode liquid chromatography

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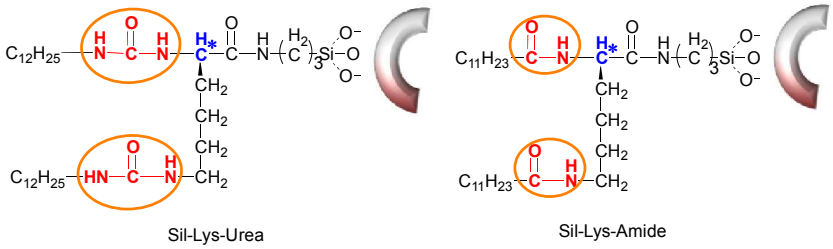
L-lysine-based, urea containing organogelator-modified silica stationary phase, with ordered functional groups, was applied for the separation of chiral analytes and polar analytes. The molecularly oriented functional group arrangement in the stationary phase enhanced the chromatographic performance thus making the separation of chiral and polar analytes possible. Chiral separation using gelator-silica hybrid particles is reported for the first time.

Chiral separation is gaining increasing interest as the majority of bioorganic molecules are chiral and exist in only one of the two possible enantiomeric forms. Chiral HPLC has proven to be one of the best techniques for the direct separation and analysis of enantiomers. Chiral separations by HPLC are achieved either directly using chiral stationary phases (CSPs) and chiral additives in the mobile phase,[1] or indirectly by the derivatization of samples.[2] Most chiral separations by HPLC are performed via direct resolution using a CSP. In this technique, the stationary phase is modified with a chiral resolving agent, and the enantiomers are separated by the formation of temporary diastereomeric complexes between the analyte and the CSP.

Different types of low molecular weight gelators (LMWGs) have been developed depending on their potential applications in sensors, electronic devices, regenerative medicine etc.[3, 4] The gelation is induced by the formation of a three-dimensional network based on highly ordered structures with a unique chirality.[5] Our group has very recently reported a glutamide derived gel-forming compound which was grafted onto silica and has studied the separation of bio-active and shape-constrained isomers.[6] Although there are detailed reports on the developments of L-lysine-based LMWGs and their properties and advantages, practical applications have not yet been developed.[7]

Herein, we report the first successful chiral separation using a gelator-silica hybrid stationary phase (Scheme 1). Chiral separation using carbamates of various polysaccharides has previously been reported,[8] but the application of a gel-forming compound in enantiomeric separation has not been reported until now. The presence of chiral centre in the L-lysine residues in the urea containing organogelator-modified silica (Sil-Lys-Urea) suggested the possible application of such particles in the separation of racemic compounds into their components, the pure enantiomers. The synthesis of the aforementioned organic phase and the modification of the silica stationary phase has been discussed by Mallik et al.[9] Chiral separation was checked in normal phase liquid chromatography (NPLC) mode. Fig. 1 shows the separation of the mixture of *(R)*- and *(S)*- (1,1'-bi-2-naphthol) (see Fig. S1, ††ESI, for analyte structure) into its constituent enantiomers using the Sil-Lys-Urea column. An amide containing the L-lysine-based phase (Sil-Lys-Amide) was used as a reference. The Sil-Lys-Urea column could separate the mixture into its constituent enantiomers, and the separation was found to be better (selectivity = 1.073) than that of the reference column (no separation was observed at all, Fig. S2, ††ESI) as well as



Scheme 1 Structure of Sil-Lys-Urea and Sil-Lys-Amide.

other chiral columns reported previously for the same analyte.[10] Such different enantioselective separation behavior is shown by two columns of the same chiral derivative. The influence of chirality is enhanced by functional group ordering which is more pronounced in urea-type derivative than in amide-type derivative. Here, functional group ordering is the outcome of intermolecular hydrogen bonding that is more concentrated in Sil-Lys-Urea, leading to more ordered stereogenic centres. Chirality is remarkably enhanced through the molecular orienting effect and the enhanced chirality induced the enantioselectivity of the stationary phase. We believe that the chiral separation using Sil-Lys-Urea column can be improved by changing the chromatographic conditions and the column can be employed to separate other chiral analytes if appropriate conditions are met.

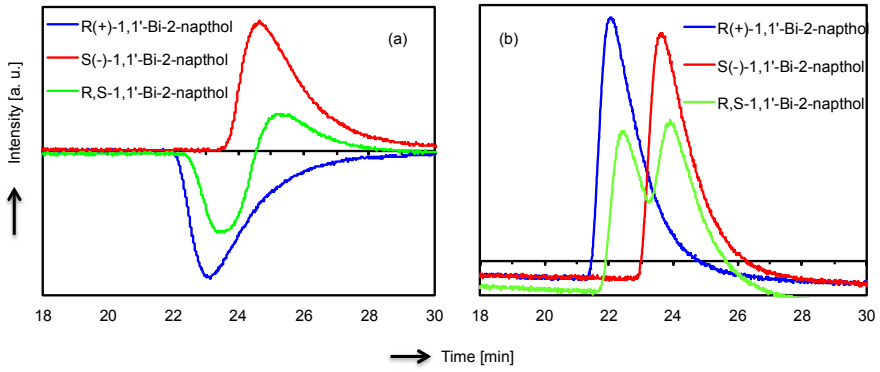


Fig.1 Separation of mixture of *R(+)*- and *S(-)*-1,1′-Bi-2-napthol into its constituent isomers with Sil-Lys-Urea column as detected by (a) CD detector and (b) UV detector. Mobile phase: hexane: tetrahydrofuran: ethanol; 70:30:0.5 at 0 °C, flow rate 1 mL min-1, λ= 254 nm.

Reversed-phase chromatography (RPPLC) is the most widely used analytical technique for the separation of complex mixtures. However, separation of polar compounds is inadequate as a highly aqueous mobile phase is required for analyte retention.[11] The effective retention of polar compounds can be achieved using NPLC but the poor solubility of polar molecules in non-aqueous mobile phases and the poor reproducibility of the method become the limiting factors.[12] Hence, hydrophilic interaction liquid chromatography[13] (HILIC), which employs a polar stationary phase like NPLC and a mobile phase like RPLC, is regarded as the best alternative for the effective separation of small polar compounds. It is a variant of NPLC that uses a reversed-phase eluent. HILIC has shown great potential, and is becoming a powerful technique in the chromatographic analysis of various kinds of hydrophilic polar compounds such as peptides, proteins, pharmaceuticals, etc.[14] The analyte retention, in HILIC, is controlled by two major mechanisms[15] namely (i) partitioning between the stagnant enriched water layer at the surface of the stationary phase and the more hydrophobic mobile phase containing 5-30% water in acetonitrile (ACN), and (ii) hydrogen bonding with the thin water layer on the stationary phase surface.[16] The high water content in the mobile phase provides significant advantages in regard to the solubility of many biologically active substances.

Further, we employed the gelator-silica hybrid stationary phase for the separation of mixture of nucleobases (see Fig. S3, ††ESI for analyte structures) and sulfur drugs (see Fig. S4, ††ESI for analyte structures) into its components using the HILIC mode. For the HILIC mode separation Sil-Lys-Amide and commercially available HILIC type amide column (see ††ESI for details) were used as references. The effect of water content on the retention behaviour of the analyte (Fig. S5, ††ESI) revealed that the retention factor decreases with an increase of water content for the selected nucleobases, which is in correspondence with typical HILIC retention characteristics.[13b, 14d] At high acetonitrile concentrations, water adsorbs more strongly on the stationary phase surface and the hydrophilic compounds are retained longer in the water-enriched layer.

The separation behaviour was studied for a mixture of eight nucleobases (Fig. 2). When using the Sil-Lys-Urea column analytes are better resolved and column shows higher selectivity compared with the reference columns. The retention time is almost double and some analytes co-elute in the case of Sil-Lys-Amide column. Though commercial amide column could separate most of the nucleobases, the retention time is very long (almost three times of the retention time for Sil-Lys-Urea column and cytidine was not eluted even after 60 minutes).

Sulfonamide antibiotics are generally used in feed additives for livestock in order to fight infections and maintain desired growth levels. Hence, the analysis of antibiotic residues in food-producing animals is important worldwide for evaluating food safety and maintaining compliance with export regulations.[17] Chromatographic separation of a mixture of eight sulfonamide antibiotics is presented in Fig. 3. All the components of the analyte are very well separated by Sil-Lys-Urea column within 15 minutes of injection. The peak resolution ranges from 2.637 to

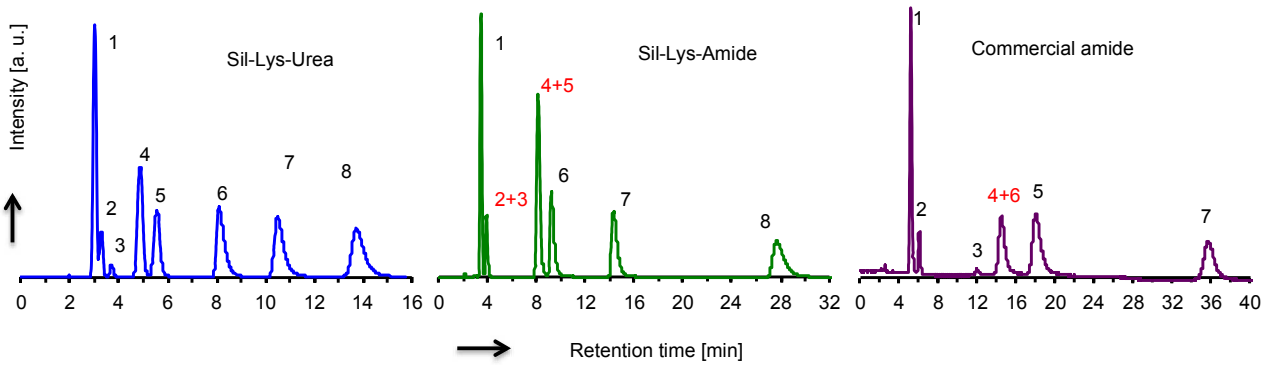


Fig. 2 Separation of nucleobases with Sil-Lys-Urea, Sil-Lys-Amide and Commercial amide column. The mobile phase is ACN: ammonium acetate buffer (95:5). The buffer concentration is 20 mM and the pH is 6.7. The analyte is the mixture of 1. Thymine 2. Uracil 3. 4, 6 diaminopyrimidine 4.Uridine 5. Adenosine 6. Adenine 7. Cytosine 8. Cytidine. Flow rate 1 mL min^{-1}, *T*= 25 ˚C, λ= 254 nm.

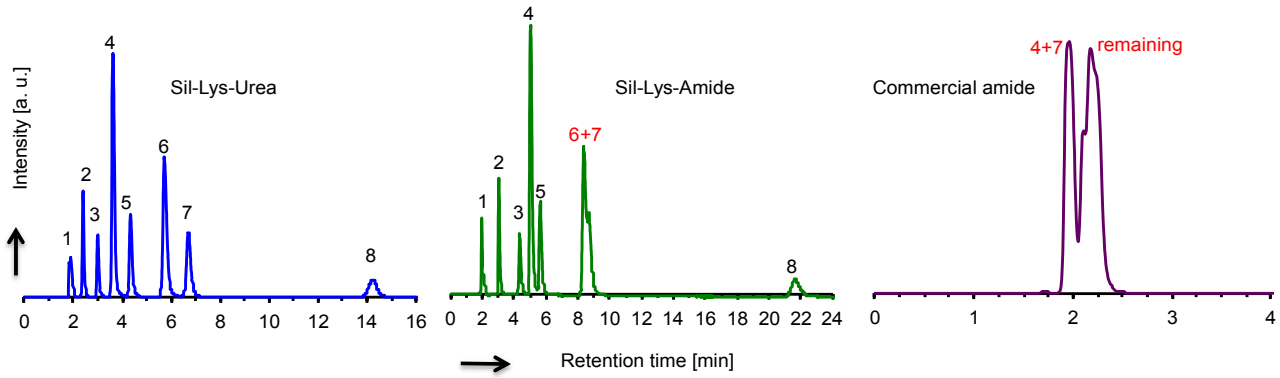


Fig. 3 Separation of sulfur based drugs with Sil-Lys-Urea, Sil-Lys-Amide and commercial amide column. The mobile phase is ACN: ammonium acetate buffer (90:10). The buffer concentration is 20 mM and the pH is 6.7. The analyte is the mixture of 1. Sulfanilamide 2. Sulfamerazine 3. Sulfadiazine 4. Sulfamethoxazole 5. Sulfathiazole 6. Sulfamonomethoxine 7. Sulfaquinoxaline 8. Sulfamethizole. Flow rate 1 mL min^{-1}, T= 25 ˚C, λ= 275 nm.

16.1. On the other hand, some of the analytes co-elute and the retention time is also longer in the case of the Sil-Lys-Amide column whereas the commercial amide column could not separate sulfonamide antibiotics as we can see the analytes co-eluting. The Sil-Lys-Urea and Sil-Lys-Amide columns differ only in the presence of the urea group in the former and the amide group in the latter. The urea group in Sil-Lys-Urea was designed as an integrated and oriented functional group (through intermolecular hydrogen bonding) with weak interaction sites to facilitate multiple-interactions with the polar analyte molecules, which could be the reason for its higher selectivity and better column performance.

To investigate the mechanism for the separation of the sulfur-based drugs and nucleobases, we studied the effect of buffer salt concentration (10-25 mM) in an acetonitrile-water mobile phase, keeping the mobile phase composition and the pH of the buffer solution constant. It is clearly seen that the retention time of sulfur drugs (Table S1, ††ESI) decreases with increasing salt concentration. The column showed lower resolution, less selectivity and less efficiency (the number of theoretical plates, *N*, decreased from the range 1253-9881 at 10 mM to 1337-6468 at 25 mM) at higher salt concentration. The elution time decreased from 26 minutes to 13 minutes on increasing the buffer salt concentration from 10 mM to 25 mM. This two-fold decrease in elution time indicates the influence of ionic interactions in the HILIC mode retention mechanism. However, in the case of nucleobases (Table S2, ††ESI), the salt concentration does not have much influence on the retention time, showing that the polar nucleobases are predominantly separated based on a partitioning mechanism. Ionic interaction has a very weak effect in the latter case.

The separation mechanism was further investigated by studying the effect of changes in the buffer pH on the chromatographic separation. At a low pH, i.e. 3.4, thymine and uracil elute together while at high pH they elute separately with resolutions 1.07 and 1.15 at pH 5.3 and 6.7, respectively. For the rest of the analytes too, both the resolution and the selectivity are lower at low pH (Fig. S6, Table S3, ††ESI). The column was most efficient at pH 6.7 with *N* ranging from 2247 to 3660.

Similar results were obtained in the case of sulfur drugs (Fig. S7, ††ESI). The column showed low resolution and less selectivity at low pH with most of the analytes co-eluting. The effect of pH is more prominent in the case of sulfur drugs than in the case of nucleobases, which further supports the fact that the sulfur drugs separate predominantly via ionic interaction whereas a partitioning mechanism plays the key role in the separation of nucleobases.

In summary, L-lysine-based urea containing organogelator-modified silica stationary phase was applied for the separation of chiral analytes and polar analyte using NPLC mode and HILIC mode, respectively. The chromatographic performance of the urea-containing column, with an ordered functional group arrangement, was found to be better than the amide-containing organogelator-modified silica stationary phase with a less ordered arrangement as well as a commercially available amide type HILIC column. The column was also employed in RPLC mode separation in the previous study,[9] which suggests that the column can be used for multi-mode chromatographic separation.

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Notes and references

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GRAPHICAL ABSTRACT